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Communication

Exopolysaccharides Produced by Phytopathogenic *Pseudomonas syringae* Pathovars in Infected Leaves of Susceptible Hosts

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ABSTRACT

Bacterial exopolysaccharide (EPS) was extracted from infected leaves of several host plants inoculated with phytopathogenic strains of *Pseudomonas syringae* pathovars. Extraction was by a facilitated diffusion procedure or by collection of intercellular fluid using a centrifugation method. The extracted EPS was purified and characterized. All bacterial pathogens which induced water-soaked lesions on their host leaves, a characteristic of most members of this bacterial group, were found to produce alginic acid (a polymer consisting of varying ratios of mannuronic and guluronic acids). Only trace amounts of bacterial EPS could be isolated from leaves inoculated with a pathovar (pv. *syringae*) which does not induce the formation of lesions with a water-soaked appearance. Guluronic acid was either present in very low amounts or absent in the alginic acid preparations. All bacterial alginates were acetylated (7–11%). Levan (a fructan) was apparently not produced as an EPS *in vivo* by any of the pathogens tested.

Bacteria in their natural environments are often surrounded by bacterial EPS[1] (2). This coating of EPS is thought to play a role in adhesion to surfaces, cation concentration, protection against adverse environmental conditions, and host resistance factors as well as other functions (2).

Many phytopathogenic bacteria are known to produce EPS *in vitro* (24); however, there have been few reports of the isolation and characterization of EPS produced by such bacteria in infected hosts. A group of leaf spotting phytopathogenic pseudomonads belonging to *Pseudomonas syringae* normally grow in the intercellular spaces and often, but not always, induce the formation of lesions with a water-soaked appearance. Members of this group of pathogens can be differentiated by host specificity and are given pathovar designations to reflect this fact (5).

Indirect evidence for EPS production in plants infected with strains of *P. syringae* pathovars comes from ultrastructural studies. Fibrillar material, which was assumed to be bacterial EPS, surrounds cells of *P. syringae* pv *coronafaciens*, pv *phaseolicola*, and pv *tabaci* in infected oat, bean, and tobacco leaf tissues, respectively (6, 13, 21). Addition of ruthenium red, a cationic dye (16), to the fixative greatly enhanced visualization of the fibrillar material indicating the anionic nature of the putative EPS (6, 21). EPS production *in vivo* has been proposed to play a role in maintaining a hydrophylic environment around the bacterial cells which is necessary for bacterial growth (7).

Studies in our laboratory (8, 19) and in that of Gross and Rudolph (9–11) have indicated that *P. syringae* pathovars when grown on a variety of carbon sources *in vitro* can produce alginic acid (a polymer consisting of varying ratios of mannuronic and guluronic acids) as an EPS. However, when sucrose is present as the primary carbon source during culture *in vitro* these bacteria produced levan (a fructan), alginic acid, or both levan and alginic acid as EPS dependent on the strain tested (8–11, 19). We previously found that strains of *P. syringae* pv *glycinea*, a pathogen of soybean, produce only alginic acid as an EPS in infected susceptible leaves even though these strains produced only levan when grown *in vitro* with sucrose as the primary carbon source (19). While this study was in progress, Gross and Rudolph (12) reported that *P. syringae* pv *phaseolicola*, a pathogen of bean, produced either levan and alginic acid in approximately equal amounts or almost exclusively alginic acid in infected bean leaf tissues dependent on the physiologic race of the bacterial strain. In this study, we have isolated and characterized the EPS produced by four additional *P. syringae* pathovars in susceptible host leaves in order to ascertain if alginic acid or levan is the predominant EPS produced and to determine if a pathovar which does not cause water-soaked symptoms on infected leaves also produces EPS *in vivo*.

MATERIALS AND METHODS

Bacteria

Pseudomonas syringae pv *lachrymans* strain PL 785 was obtained from C. Leben; *P. syringae* pv *phaseolicola* strains R1 from A. W. Saettler, R2 from D. J. Hagedorn, At from D. M. Webster; *P. syringae* pv *syringae* strains Meyer from D. M. Webster and CFBP 1542 from R. Samson; and *P. syringae* pv *tomato* strain 84-86 from R. D. Gitaitis. Bacteria were maintained on *Pseudomonas* agar F (Difco Laboratories) at 4°C with monthly transfer. Long-term storage was by lyophilization.

[1] Abbreviations: EPS, exopolysaccharide; CFU, colony-forming units; MATMAB, mixed alkyltrimethylammonium bromide.

Plant Materials and Inoculations

Seed of bean (*Phaseolus vulgaris*) cv Red Mexican UI 34 were kindly supplied by P. Lindgren. Seed of bean cvs Red Kidney and Improved Tendergreen (W. Atlee Burpee Co., Warminster, PA), cucumber (*Cucumis sativus* L.) cv Ashley and tomato (*Lycopersicon esculentum* Mill) cv Better Boy VFN (Crosman Seed Co., East Rochester, NY) were cultivated in Baccto potting soil (Michigan Peat Co., Houston, TX) in plastic flats. Plants were maintained in a growth chamber at 26°C day, 20°C night, 75% RH. Fluorescent and incandescent bulbs provided 1.1×10^4 lux on a 13 h photoperiod.

For the isolation of EPS from infected plant material, leaves of young plants were inoculated by forcibly spraying their abaxial surfaces with bacterial suspensions using a chromatographic spray bottle until the tissue had a water-soaked appearance. Inoculum was prepared by suspending bacterial cells from overnight cultures grown on *Pseudomonas* agar F at 28°C in water to give an A_{600} nm of 1.0. This suspension was diluted 10-fold with water to give an inoculum concentration of approximately 5×10^6 CFU per mL based on a standard curve of A_{600} nm *versus* bacterial CFU per mL generated by standard dilution plating techniques. After all evidence of spray induced water-soaking disappeared (approximately 2 h), inoculated plants were returned to the growth chamber. Controls consisted of leaves sprayed with water alone. Only bean cv Red Kidney was used for determining *in vivo* EPS production by bean pathogens.

For determination of physiologic race, leaves of differential bean cv Red Kidney (susceptible to races 1, 2, and 3), cv Red Mexican UI 34 (susceptible to races 2 and 3, resistant to race 1) and cv Improved Tendergreen (susceptible to races 1 and 2, resistant to race 3) were forcibly sprayed with inoculum containing approximately 5×10^7 CFU/mL.

Isolation and Purification of EPS

Three to 7 d after inoculation, inoculated leaves were detached from the plants, weighed, and the bacterial EPS produced *in vivo* extracted. Two methods of extraction to obtain crude extracts were utilized. The first was a facilitated diffusion procedure as previously described (19). Briefly, detached leaves were vacuum infiltrated with water containing an antibiotic and left to stand overnight at 4°C. The liquid remaining after removal of the leaves and subsequent clarification by centrifugation was lyophilized. In the second method, detached leaves were vacuum infiltrated with water, the infiltrated leaves were collected, blotted dry with paper towels, midveins were removed with a scalpel, and the intercellular fluid was immediately obtained by centrifugation according to the method of Klement (15). Leaf halves were centrifuged three times for 45 min at 23,300*g* each time. Intercellular fluid which collected on the bottom of the centrifuge bottles was removed after each run, pooled, and lyophilized.

Partial purification of the extracted crude EPS was by gel permeation chromatography (ACA-202 [LKB Instruments, Inc.]; fractionation range, 1×10^3 to 15×10^3) to remove low mol wt contaminants (19). The protein content of these EPS samples was then reduced by extraction with cold buffered phenol (14). Next, samples were subjected to ultracentrifugation (100,000*g*, 4 h). Both the resultant pelleted material and the supernatant fluids were collected and lyophilized. Alternatively, EPS samples were partially purified by the method of Sutherland (22) with the primary purification steps being acetone precipitation, ultracentrifugation (100,000*g*, 4 h) and then acetone reprecipitation.

As a final purification step for material obtained by either extraction procedure, two methods were tested for separation of neutral polysaccharides present in the EPS preparations. The first utilized ion-exchange chromatography. EPS samples were taken up in 2 or 3 mL of 0.05 M Tris-HCl buffer (pH 8.0) to give solutions containing approximately 20 mg/mL. Insoluble material was removed by centrifugation and the samples were then loaded onto a column (12 × 1.7 cm) of DEAE-Sepharose CL-6B (Pharmacia, Inc., Piscataway, NJ) equilibrated with buffer. The column was washed with two bed volumes (bed volume = 20 mL) of buffer to elute any neutral polysaccharides present followed by buffer containing 1 M NaCl for elution of acidic EPS. Fractions were dialyzed against distilled water and lyophilized. Alternatively, EPS was dissolved in water at 5 mg/mL and an equal volume of 1% aqueous MATMAB (Sigma) was added with stirring. The resultant precipitate was collected by centrifugation and the pelleted material was taken up in 1 M NaCl to dissolve the EPS-MATMAB complex. The free EPS was precipitated several times with acetone (3 volumes, −20°C). The resultant EPS preparation and the non-MATMAB precipitable material, which had been dialyzed against distilled water to remove MATMAB, were lyophilized.

Characterization of EPS

All reagents were supplied by the Sigma Chemical Co. Protein content was determined by a modified Lowry method (17) with BSA as standard. Total neutral sugar was determined by reaction with phenol-sulfuric acid (4) with glucose as the standard, and uronic acid content was determined by reaction with *m*-hydroxybiphenyl (1) with D-mannurono-6,3-lactone as the standard. Acetyl content was determined by reaction with hydroxylamine hydrochloride (18) with glucose pentaacetate as standard.

Sugar content of the samples was determined by GLC using a Hewlett-Packard 5880 gas chromatograph fitted with a 15-m SP-2330 capillary column. The column was temperature programmed from 125 to 225°C at 4°C/min. EPS samples high in uronic acid based on colorimetric assay were reduced with sodium borohydride via the carbodiimide adduct (23, 25) as described previously (19) before hydrolysis and derivatization. All samples were hydrolyzed in H_2SO_4 at 100°C for 90 min except when the presence of levan was to be determined. In this case, samples were hydrolyzed in 1 M oxalic acid at 70°C for 90 min. After neutralization, sugars were characterized as their aldononitrile acetate derivatives as described by Varma *et al.* (26) or, in the case of levans, as their acetate derivatives. Acetate derivatives were prepared by heating samples (2 mg) in pyridine (200 μL)-acetic anhydride (150 μL) at 70°C for 90 min.

RESULTS AND DISCUSSION

Inoculation of the differential bean cultivars indicated that *Pseudomonas syringae* pv *phaseolicola* strain R1 was of phys-

iologic race 1, while strains R2 and At were of physiologic race 2.

At the time of harvest, all leaves inoculated for the purpose of isolating bacterial EPS produced *in vivo* had extensive water-soaked lesions present except for bean leaves inoculated with *P. syringae* pv *syringae* strains Meyer and CFBP 1542. These two interactions resulted in the formation of extensive brown lesions without a water-soaked appearance as typical of the bacterial brown spot disease of bean caused by this bacterium. Control leaves showed no symptoms.

The efficiency of the overnight facilitated diffusion extraction method and the centrifugation method were tested in single experiments using the interactions *P. syringae* pv *phaseolicola* strain R1 or strain At *versus* bean, respectively. After the initial extraction procedure, leaves were homogenized in water with a commercial blender. The homogenization step resulted in the extraction of additional crude material with 26 and 493% of the dry weight of the material initially extracted by the diffusion or the centrifugation methods, respectively. After final purification, this additional crude material yielded an additional 7.4% (initial diffusion method) to 24% (initial centrifugation method) of EPS.

For bacteria (*P. syringae* pvs *phaseolicola, lachrymans,* and *tomato*) which caused water-soaking of susceptible host leaf tissues, the amount of crude material extracted by the facilitated diffusion method ranged from 23.7 to 54.3 mg dry weight/g fresh weight leaf (Table I). Much less crude material was extracted from the water-sprayed control leaves by this method (Table I). Use of the centrifugation method resulted in the extraction of considerably reduced amounts of crude material for both infected and control leaves compared to the facilitated diffusion method (Table I). Samples obtained after gel permeation chromatography contained high levels of protein (15–50%) which was reduced to 5% or less by a single extraction with cold buffered phenol. Initial purification of crude material by the method of Sutherland (22) led to samples containing less than 5% protein. Based on colorimetric assay, significant amounts of uronic acid (>20%) were present only in the partially purified samples originating from leaves inoculated with bacteria. At this stage of purification, samples originating from water-sprayed control leaves contained 6% or less uronic acid.

As levan, but not alginate (8), sediments during ultracentrifugation at 100,000*g*, we examined all such pelleted material for the presence of levan. Sedimented material usually represented less than 5% of the total sample dry weight before ultracentrifugation. Based on analysis by GLC, no levan was present in any of the samples.

Before removal of neutral sugars was attempted, all samples of nonsedimentable, high mol wt material from leaves inoculated with bacteria (except for bean leaves inoculated with *P. syringae* pv *syringae* strain CFBP 1542 due to the very limited amount of material available) were examined for the presence of alginic acid by GLC. Analysis of unreduced samples indicated that arabinose and galactose were the predominant neutral sugars present. Lesser amounts of glucose, mannose, rhamnose, and xylose were also present in all samples, while fucose was occasionally detected. Equivalent samples from water-sprayed leaves showed similiar patterns of neutral sugar composition. After reduction of the samples from infected leaves, only mannose showed a significant increase (up to 333-fold) in peak area relative to arabinose. Relative peak areas for galactose in reduced samples increased from 22 to 323% over those in unreduced samples. No other sugars showed an increase in peak area relative to arabinose after sample reduction. Low levels of glucose were found in some of the reduced samples. These findings indicated that the uronides present in the samples from infected leaves were

Table I. *Exopolysaccharide Production by P. syringae Pathovars in Vivo*

Host	Inoculum	Isolation Procedure[a]	Crude Material	Purified Alginate	Alginate Composition	
					Guluronate[b]	Acetate[c]
			mg dry wt/g fresh wt leaf		%	
Bean	H_2O	FD	9.0			
		C	0.8			
	P. syringae pv *phaseolicola*					
	Strain R1	FD	23.7	7.3	5	ND[d]
		C	5.8	4.7	0	11
	Strain R2	FD	24.8	0.9	4	9
	Strain At	FD	44.1	3.0	0	7
		C	1.2	0.4	4	9
	P. syringae pv *syringae*					
	Strain Meyer	C	1.6	Tr[e]	0	ND
Cucumber	H_2O	FD	8.4			
	P. syringae pv *lachrymans*					
	Strain PL 785	FD	33.8	1.8	0	8
Tomato	H_2O	FD	19.9			
	P. syringae pv *tomato*					
	Strain 84–86	FD	54.3	1.5	0	9

[a] FD, facilitated diffusion method; C, centrifugation method. [b] % guluronate based on GLC analysis, guluronate/(mannuronate + guluronate) × 100. [c] % acetate determined by colorimetric assay. [d] Not determined. [e] Trace (<0.1 mg dry wt/g fresh wt).

predominantly alginic acid along with a lesser amount of polygalacturonide.

Next, two methods for the removal of neutral sugars from the alginate preparations were tested. Ion-exchange chromatography on DEAE-Sephadex CL-6B was only partially successful in removing the neutral polysaccharides from the samples, while almost complete removal of neutral polysaccharides was obtained by a single precipitation with MATMAB.

After final purification by removal of neutral sugar by precipitation with MATMAB, GLC analysis of reduced alginate samples indicated that mannose accounted for >90% of the sugars present along with variable amounts of gulose (Table I). Yields of alginate from leaves inoculated with bacteria which induced a water-soaked appearance ranged from 0.4 to 7.3 mg dry weight/g fresh weight leaf tissue (Table I). The purified alginates were acetylated (7–11%) (Table I) as is common for bacterial alginates (3). Bean leaves inoculated with either of the two *P. syringae* pv *syringae* strains which do not induce water-soaking yielded only trace amounts of purified material (<0.1 mg dry weight/g fresh weight) (Table I). The identification of the purified material as alginate by GLC analysis was done only for *P. syringae* pv *syringae* strain Meyer.

The results of this and previous studies (8, 12) clearly demonstrate that strains of phytopathogenic *P. syringae* pathovars which induce water-soaked lesions on their host leaves do produce the acidic EPS alginic acid *in vivo*. Alginic acid produced *in vivo* by *P. syringae* pathovars appears to be uniformly low or lacking in guluronic acid similar to the alginates produced *in vitro* by these bacteria (8, 10). The ability to produce alginic acid *in vivo* is probably common to all members of this group which induce lesions with a water-soaked appearance. In the present study, we have determined that one *P. syringae* pathovar (pv *syringae*) that does not induce the formation of water-soaked lesions on its host appears to produce very low amounts of alginate *in vivo*. This may indicate that production of alginate *in vivo* is important either for the induction of water-soaking of host tissues or the maintenance of the water-soaked condition, or both.

Two strains used in this study (*P. syringae* pv *lachrymans* PL 785 and *P. syringae* pv *tomato* 84–86) were previously found in our laboratory to be incapable of producing alginate *in vitro* in a liquid medium (8). The fact that these two strains did produce alginic acid in their host indicates that the genes responsible for alginic acid synthesis and transport are present in these two strains but are only expressed under appropriate conditions as provided in the host intercellular spaces.

Levan production by *P. syringae* pathovars in infected leaves appears to be uncommon. Gross and Rudolph (12) recently reported that a race 1 strain of *P. syringae* pv *phaseolicola* produced approximately equal amounts of levan and alginic acid in susceptible bean leaves, while a race 2 strain produced predominantly alginic acid. Such race specific differences in the EPS produced by this pathovar *in vivo* were not substantiated by our study. Why bacterial strains capable of levan production *in vitro* often do not appear to produce levan *in vivo* even though sucrose is the main translocatable sugar present is not yet clear. Possibly *de novo* synthesis or activation of host cell invertase or sucrose synthase enzymes (20) occurs upon infection whose action converts sucrose to its constituent sugars glucose and fructose before appreciable amounts of sucrose can enter into the intercellular spaces and become available for utilization by the bacteria.

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